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Circulating tumor DNA as a non-invasive substitute to metastasis biopsy for tumor genotyping and personalized medicine in a prospective trial across all tumor types

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ABSTRACT

Cell-free tumor DNA (ctDNA) has the potential to enable non-invasive diagnostic tests for personalized medicine in providing similar molecular information as that derived from invasive tumor biopsies. The histology-independent phase II SHIVA trial matches patients with targeted therapeutics based on previous screening of multiple somatic mutations using metastatic biopsies. To evaluate the utility of ctDNA in this trial, as an ancillary study we performed *de novo* detection of somatic mutations using plasma DNA compared to metastasis biopsies in 34 patients covering 18 different tumor types, scanning 46 genes and more than 6800 COSMIC mutations with a multiplexed next-generation sequencing panel. In 27 patients, 28 of 29 mutations identified in metastasis biopsies (97%) were detected in matched ctDNA. Among these 27 patients, one additional mutation was found in ctDNA only. In the seven other patients, mutation detection from metastasis biopsy failed due to inadequate biopsy material, but was successful in all plasma DNA samples providing three more potential actionable mutations. These results suggest that ctDNA analysis is a potential alternative and/or replacement to analyses using costly, harmful and lengthy tissue biopsies of metastasis, irrespective of cancer type and metastatic site,

Abbreviations: ctDNA, circulating cell-free tumor DNA; cfDNA, circulating cell-free DNA; NGS, next-generation sequencing.

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for multiplexed mutation detection in selecting personalized therapies based on the patient's tumor genetic content.

1. Introduction

Personalized medicine in oncology proposes the customization of healthcare using molecular analysis. In this context, diagnostic testing is used to select appropriate and optimal therapies based on a patient's cancer genome. Circulating cell-free tumor DNA (ctDNA) has significant potential as a biomarker in oncology (Bidard et al., 2013; Crowley et al., 2013). Detection of ctDNA in cancer patients after curative treatment indicates the presence of minimal residual disease and is likely to be a prognostic marker of relapse (Diaz and Bardelli, 2014; Schwarzenbach et al., 2011). In a proof of concept study, early ctDNA changes during treatment were associated with breast cancer response to treatment (Dawson et al., 2013). These first ctDNA applications were developed with assays targeting mutation(s) previously identified by genomic characterization of each patient's tumor, the biopsy of which may not be available. Thus, another potential use of ctDNA is the *de novo* detection of somatic mutations, particularly those shown to be predictive of response or resistance to targeted therapy, thereby substituting solid tissue biopsies as a source of tumor DNA. Clinical validation for *de novo* mutation detection using plasma has been provided, but only for a restricted set of mutations in *KRAS* and/or *BRAF* linked to a particular histology, colorectal cancer (Bettegowda et al., 2014; Thierry et al., 2014). This analysis did not cover the variety of actionable mutations for targeted therapy across multiple cancer types.

In a selected patient population with unusually high ctDNA levels, wide coverage exome next-generation sequencing (NGS) detected ctDNA mutations appearing at the time of treatment resistance (Murtaza et al., 2013). However, a significant proportion of biopsy mutations were not found in plasma. This contrasts with a metastatic ovarian study that reported recovery of most tumor mutations from plasma using more limited, but still multiplexed NGS (Forshew et al., 2012). Therefore, the clinical validity of using a multi-region assay on ctDNA to detect a range of drug actionable mutations *de novo* requires additional evidence, particularly across multiple cancers.

The ongoing large multicentric randomized histology-independent phase II trial (SHIVA, NCT01771458; Le Tourneau et al., 2014) provides a unique opportunity to test whether *de novo* ctDNA analysis matches the mutation analysis of tumor tissue. This trial compares molecularly targeted therapy based on tumor molecular profiling versus conventional therapy in patients with any type of refractory cancer. The screening phase consists of the invasive biopsy of metastatic tumor tissue and downstream analysis using Ion Torrent's Ampliseq hotspot cancer panel. In a proof-of-principle analysis, we evaluated whether ctDNA analysis would identify the same mutations as those obtained through invasive biopsy. Therefore, we prospectively collected and analyzed by NGS fresh plasma samples in patients who underwent invasive biopsy as part of the trial screening procedure.

2. Methods

2.1. Patient and sample collection

All patients were included after written informed consent. Patients older than 18 years with any type of recurrent and/or metastatic cancer who failed standard therapy were eligible for the study provided their disease was measurable and accessible for a biopsy or resection of a metastatic site. Patients were allowed to receive conventional chemotherapy between the biopsy and the time of randomization, when blood samples were collected in two 10 mL EDTA tubes. For 3/34 of patients, P-16, P-27, and P-28, the clinical protocol was not adhered to and targeted therapy was provided before randomization. The targeted therapeutics used are given in Table 1 below. The main trial and ancillary studies were approved by the ethics committee and the French "Agence nationale de sécurité du médicament et des produits de santé" in September 2012. Funding sources played no role in study design, collection, analysis and interpretation of data, in the writing of the report; and in the decision to submit the article for publication.

2.2. Metastasis biopsy and plasma DNA extractions

DNA was extracted from the fresh frozen biopsy using the QiaAmp® nucleic acid kit (Qiagen). Cellularity was determined on the tissue sections using standard clinical protocols. Only frozen biopsies with $\geq$30% tumor cells were processed for DNA extraction and NGS. In parallel, blood was centrifuged for plasma extraction as described and as previously performed (Diehl et al., 2008; Madic et al., 2012). Briefly, EDTA-tubes were centrifuged for 10 min at 820 g within 3 h of the blood draw. Supernatants were further centrifuged at 16,000 g for 10 min at 15 °C to remove debris. Plasma was harvested and stored at −80 °C until needed. When DNA was to be analyzed, ~2 mL plasma was thawed and ctDNA extracted using the QIAamp® Circulating Nucleic Acid (Qiagen). DNA from both metastasis biopsies and plasma samples were quantified with Qubit (Life Technologies) accordingly to the manufacturer's protocol and stored at −20 °C before use.

2.3. Mutation screening

Screening of mutations was performed by targeted sequencing using the Ion Ampliseq Hotspot cancer panel V1 in conjunction with the AmpliSeq library kit v2.0 and the Ion

Table 1 – Patients characteristics.

Patient	Age (years)	Tumor type	Number of metastatic sites	Tumor biopsy site	Length of stay (nights)	% tumor cells in biopsy	Time diff. biopsy – plasma (days)	Treatment between biopsy and plasma
P-1	65	Ovarian ADC	5	Lymph node	0	50%	152	Carboplatine + Gemcitabine
P-2	70	Breast ADC	3	Liver	1	50%	224	Paclitaxel
P-3	62	Breast ADC	2	Lymph node	2	80%	258	Eribuline then Gemzar
P-4	66	Breast ADC	4	Skin	0	<30%	41	Paclitaxel
P-5	47	Cervix SCC	2	Lymph node	3	70%	254	Capecitabine
P-6	69	Breast ADC	3	*Biopsy failure*	1	–	215	Cyclophosphamide then Paclitaxel
P-7	65	Anal SCC	4	Lymph node	3	80%	40	Docetaxel
P-8	67	Lung ADC	4	Lung	1	90%	271	Docetaxel
P-9	61	SCLC	2	*Biopsy failure*	1	–	244	Topotecan
P-10	68	Lung ADC	1	Lymph node	1	70%	139	None
P-11	66	Endometrial ADC	2	Lymph node	2	70%	494	Cyclophosphamide
P-12	53	Breast ADC	2	Liver	1	<30%	180	Eribuline then Etoposide
P-13	79	Bladder carcinoma	1	Bladder	1	95%	96	Vinorelbine
P-14	59	ACUP	1	Liver	1	80%	179	None
P-15	69	Melanoma	3	Liver	1	90%	53	None
P-16	58	Adenoid cystic carcinoma	3	Liver	1	70%	175	Erlotinib then Gemcitabine
P-17	55	H&N SCC	3	Lymph node	1	70%	112	Methotrexate then Vinorelbine
P-18	61	Endometrial ADC	2	Vagina (pelvectomy)	17	50%	112	None
P-19	65	Lung ADC	2	Liver	1	50%	123	Docetaxel then Gemcitabine
P-20	68	Lung ADC	3	Lung	0	<30%	166	Gemcitabine
P-21	62	H&N SCC	1	Skin	0	80%	124	Paclitaxel
P-22	62	Ovarian ADC	2	Stomach	1	70%	203	Paclitaxel
P-23	48	Gastric ADC	3	Stomach	0	30%	55	None
P-24	69	H&N SCC	2	Nasal cavity	0	40%	46	Methotrexate
P-25	72	Cervix SCC	1	Lymph node	9	50%	155	Carboplatin + Paclitaxel
P-26	69	Esophagus ADC	3	Liver	1	60%	126	Irinotecan + 5 FU
P-27	70	Colorectal ADC	2	Liver	1	50%	55	Regorafenib
P-28	49	Colorectal ADC	4	Vagina	1	50%	53	Regorafenib
P-29	85	Thyroid Carcinoma	3	Thyroid	0	<30%	9	None
P-30	67	Gastric ADC	2	*Biopsy failure*	0	–	15	None
P-31	68	Pancreatic ADC	3	Peritoneum	1	30%	5	None
P-32	52	Esophagus SCC	2	Lymph node	1	90%	0	None
P-33	77	Esophagus ADC	2	Liver	1	30%	0	None
P-34	63	Pancreatic ADC	3	Liver	1	70%	0	None

ACUP: adenocarcinoma of unknown primary; ADC: adenocarcinoma; H&N: head and neck; SCC: squamous cell carcinoma; SCLC: small cell lung cancer.

Torrent Personal Genome Machine (PGM, Life Technologies). Samples at later dates were processed with the updated V2 panel. The broader coverage of the V2 panel was truncated bioinformatically to match that of the V1 panel. For the Ampliseq panel 1, the median amplicon length was 67 bp (range [44–121]) and for panel 2, the median was 109 bp (range [50–141]). Since ctDNA length is similar to that of formaldehyde-fixed paraffin-embedded (FFPE) DNA (Newman et al., 2014), ctDNA was analyzed by the same procedures as for tissue DNA, following manufacturer's instructions. At least five nanograms of extracted DNA was used to generate amplicons in 46 oncogenes and tumor suppressor genes through optimized multiplex PCR. After PCR enrichment, amplicon extremities were partially digested and Ion adapters, including one with a molecular barcode, were ligated at both ends. After limited cycle PCR of the library, quality control was carried out on a BioAnalyzer (Agilent) or a Labchip GX (Perkin Elmer) and quantified with Qubit (Life Technologies). Template preparation was performed using the Ion OneTouch System (Life Technologies) with the Ion OneTouch 200 Template Kit v2 DL (Life Technologies). Templates were sequenced on Ion Torrent PGM according to manufacturer instructions. The overall quality of each run was evaluated based on the report generated by the Torrent Server. Quality filters for downstream bioinformatics analysis included: 1) At least 100,000 reads per sample and 2) 99% of targeted positions covered at 1×, 97% at 20×, and 95% at 100× (data generated by Torrent Suite). The median read lengths were 79 and 105 bp for samples enriched with Ampliseq panels 1 and 2, respectively.

2.4. *Mutation analysis*

The bioinformatics pipeline used to perform variant calling for the biopsy-derived DNA on AmpliSeq cancer panel from PGM sequencing was done as described previously (Servant et al., 2014). For ctDNA samples analyzed here, variant calling was performed in a blinded-fashion using the exact same bioinformatics pipeline as for the metastasis biopsy sample and the same allelic frequency threshold (>1%) to call a mutation. Briefly, raw reads were aligned on the reference human genome hg19 using the TMAP aligner (v0.3.7 Life Technologies). Variant calling was carried out on the mapped reads by Torrent Variant Caller (v4.0 Life Technologies). The variants were annotated using ANNOVAR (version 2013/10/25) and the following databases: COSMIC68, dbSNP137, 1000 genomes, ESP6500, and RefGene annotations. Known PGM-related issues in homopolymer regions (Tarabeux et al., 2014) were filtered. 85% of the aligned reads extended the length of the amplicon whilst 15% of reads were truncated. Using Integrative Genomics Viewer (IGV) version 2.0.35 (Thorvaldsdottir et al., 2013), variants only observed on truncated reads were filtered out. A filter for strand bias was applied whereby variations with <20% contributed from either strand only were removed from the dataset. To report the coverage and the variant frequency in the dataset, DepthOfCoverage function was used from the Genome Analysis Toolkit (GATK v.1.6-5) software package, and mapping quality filtering of 8 and base quality filtering of 17 based on Life Technologies recommendations. Only variants covered >50× were retained.

Only non-synonymous variants characterized by a COSMIC ID (v69) and not observed in >1% of the population (1000 genomes and ESP6500) are identified as possible targetable mutations. According to this filter, 6892 possible mutations are found in the AmpliSeq Cancer panel V1 and 8927 on V2. The AmpliSeq Cancer panel V2 design covered all V1 mutations and additional ones. For this study, the additional region covered by V2 was parsed bioinformatically so that V1 and V2 were matched.

Among all mutations covered in the AmpliSeq Cancer panels, several ones were defined as "actionable" according to mutations for which a drug is available and included in the clinical study. The algorithm for this is described in a previous publication (Le Tourneau et al., 2014).

3. Results

At the time of the present ctDNA study, 741 patients were enrolled in the SHIVA trial. Of these patients, approximately 200 were randomized. Plasma samples were collected for approximately 100 of these patients, with 34 of those samples located at Institut Curie. All 34 samples were included in this feasibility analysis, covering 18 tumor types. Other than a plasma sample being available at Institut Curie, no other screening criteria were applied to select these samples for our study. Clinical characteristics of analyzed patients are shown in Table 1, together with sites of biopsy and length of the hospital stay for the biopsy. Sequencing results of solid biopsies were not feasible for seven patients, mainly due to insufficient cellularity (less than 30%). More than five nanograms of DNA were extracted from 2 mL plasma in all cases (Table 2). Median ctDNA amount was 27.4 ng/mL [range: 5.8–423]. 3.8–10 ng of plasma DNA was subjected to AmpliSeq Ion Torrent sequencing. Among the different samples, we obtained a median coverage of 1075× (range [371–2355]) and 2800× (range [295–4545]) for plasma and solid biopsies, respectively. Per amplicon, median coverages of 2217× for solid tumor biopsies and 918× for ctDNA were obtained.

Mutation detection results are shown for each patient in Table 2. In the 27 patients with NGS results, solid biopsy sequencing identified at total of 29 mutations including 26 unique potentially actionable mutations in *TP53*, *PIK3CA*, *MLH1*, *SMAD4*, *STK11*, *BRAF*, *FBXW7*, *MET*, *HRAS* and *KRAS*. All mutations identified were substitutions. Strikingly, 28 of these 29 mutations (97%) were retrieved by a blinded analysis of plasma DNA (Table 2) and one additional mutation in *APC* was found in the liquid biopsy only.

Among the seven patients with no available result from the solid biopsy, three patients displayed one actionable mutation in each respective plasma DNA, in *PIK3CA* and *TP53*. In patients with detectable plasma mutations, tumor DNA represented on average 25% of the total cell-free circulating DNA (cfDNA) fraction (range [1.2%–80%]) as determined by NGS variant percentages of wild-type alleles (Table 2). No correlation was observed between ctDNA and total plasma DNA quantity. As a control, analysis of background local error rate in all non-mutated plasma samples for those mutations

Table 2 – Mutations called in tumor biopsy and in plasma.

Patient	Extracted cfDNA (ng/mL plasma)	cfDNA sequenced (ng)	Mutation	% variant		Detected (>1% variant) biopsy/plasma	Concordance biopsy-plasma (Y/Y or N/N)
				Metastasis biopsy (reads)	Plasma (reads)		
P-1	6.8	4.5	*TP53* p.P278S	31.5 (5225)	31.3 (1677)	Y/Y	Yes
P-2	138.1	10	*PIK3CA* p.E545K	71.8 (241)	68.7 (527)	Y/Y	Yes
P-3	67.5	10	None	–	–	N/N	Yes
P-4	18	10	None	Not available	–	NA/N	–
P-5	30.8	10	*MLH1* p.R389Q	76.2 (4954)	56.5 (1179)	Y/Y	Yes
			ALK intronic variant	46.8 (3123)	45 (384)	Y/Y	Yes
			PIK3CA p.E545K	45.9 (135)	14.9 (215)	Y/Y	Yes
			SMAD4 p.S178X	17.6 (2284)	3.3 (391)	Y/Y	Yes
P-6	185.4	10	*PIK3CA* p.H1047R	Not available	26.7 (644)	NA/Y	–
P-7	14.1	10	*PIK3CA* p.E545K	17.7 (62)	23.3 (348)	Y/Y	Yes
P-8	40.2	10	*STK11* p.D194Y	36.2 (2426)	1.6 (985)	Y/Y	Yes
			TP53 p.V173L	36.5 (1391)	1.2 (2060)	Y/Y	Yes
P-9	11.1	7.4	*TP53* p.C275F	Not available	7.4 (780)	NA/Y	–
P-10	147.3	10	*APC* p.R1463R	0.13 (777)	1.9 (265)	N/Y	No
			TP53 p.R249M	53.1 (2654)	1.2 (2345)	Y/Y	Yes
P-11	17.1	10	None	–	–	N/N	Yes
P-12	43.2	10	*TP53* p.E271V	Not available	3.7 (410)	NA/Y	–
P-13	241.2	10	*PIK3CA* p.T1025A	57.6 (3648)	12.1 (511)	Y/Y	Yes
			TP53 p.R175H	60 (363)	2.2 (413)	Y/Y	Yes
P-14	5.8	3.8	*KRAS* p.Q61H	30.4 (1881)	0.4 (1260)	Y/N	No
			ALK intronic variant	46.2 (1658)	45.7 (707)	Y/Y	Yes
P-15	24.1	10	*BRAF* p.N581T	62.0 (4491)	26.3 (543)	Y/Y	Yes
P-16	11.2	7.4	None	–	–	N/N	Yes
P-17	423	10	None	–	–	N/N	Yes
P-18	21.3	10	*FBXW7* p.S396S	51.7 (2671)	46.4 (1755)	Y/Y	Yes
			PIK3CA p.H1047R	25.3 (4696)	3 (1894)	Y/Y	Yes
			TP53 p.S241F	36.6 (3856)	2.4 (4182)	Y/Y	Yes
P-19	33	10	*TP53* p.R156P	48.5 (1688)	17 (1715)	Y/Y	Yes
P-20	54	10	None	Not available	–	NA/N	–
P-21	83.9	10	None	–	–	N/N	Yes
P-22	82	10	TP53 p.V157G	72.2 (883)	42.1 (682)	Y/Y	Yes
P-23	37.3	10	*TP53* p.G266E	20.4 (928)	48.4 (888)	Y/Y	Yes
P-24	17.8	10	*MET* p.R988C	55.4 (2619)	45.8 (2014)	Y/Y	Yes
			HRAS p.A59A	40.8 (1585)	49.8 (908)	Y/Y	Yes
P-25	13	8.6	*PIK3CA* p.E545K	28 (721)	3.8 (339)	Y/Y	Yes
P-26	34.9	10	None	–	–	N/N	Yes
P-27	131.4	10	*PIK3CA* p.E545K	47.6 (313)	16.4 (268)	Y/Y	Yes
			KRAS p.G12A	47.8 (2846)	22.5 (777)	Y/Y	Yes
P-28	17.8	10	*SMARCB1* intronic variant	49.5 (2547)	46.6 (824)	Y/Y	Yes
P-29	9.8	6.5	None	Not available	–	NA/N	–
P-30	20.9	10	None	Not available	–	NA/N	–
P-31	13	8.6	None	–	–	N/N	Yes
P-32	144	10	*TP53* p.V157F	89 (399)	79.9 (812)	Y/Y	Yes
P-33	18	10	*TP53* p.R273H	2.64 (1476)	5.4 (368)	Y/Y	Yes
P-34	22.2	10	None	–	–	N/N	Yes

NA: not available (sequencing not performed due to biopsy failure or low tumor cellularity).

considered actionable in patients included in our study confirmed that the 1% allelic frequency threshold was adequate for calling the mutations identified here (Figure 1). Synthetic reconstitution experiments using cell lines mutated for *KRAS* c.345G>T and *BRAF* c.1799T>C, respectively, confirmed that when no variant DNA was added, the observed variant frequency did not approach the 1% threshold (Supplementary Figure S1). Of note, in the main SHIVA trial, a 4% threshold was set since low frequency variants were considered as not informative to select a targeted therapy. Thus, some mutations reported here are not annotated in the main trial.

4. Discussion

Precision medicine is defined as the delivery of individually adapted medical care based on the genetic characteristics of each patient and his/her tumor (Arnedos et al., 2014). This requires high-throughput technologies such as microarrays and

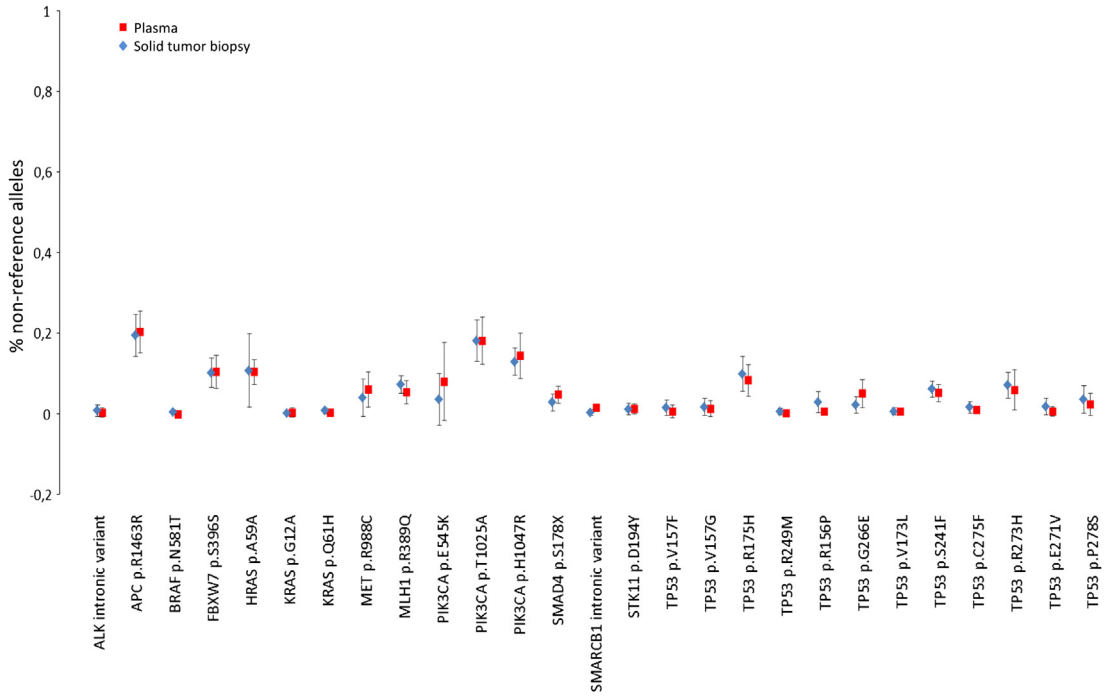

Figure 1 – Average non-reference allelic frequencies in non-mutated plasma samples. The background error rate of the whole procedure for those mutations called and considered as having actionable information in the clinical study. Error bars represent 99% confidence intervals, supporting 1% allelic frequency as an appropriate threshold to call a mutation in plasma.

NGS. Several personalized medicine programs consisting of profiling the tumor samples from each patient, identifying key oncogenic drivers, and treating the patient accordingly have shown the feasibility of this approach. Of note, anti-tumor therapeutic effects based on the tumor molecular profile are currently modest (Andre et al., 2014). Since changes in biological features between the primary and secondary tumors can occur, the molecular characterization of the tumor is based on the biopsy of metastatic disease. However the acquisition of tissue from metastatic deposits is a challenge for these trials because of the invasiveness of the procedure and the unreliable outcome of biopsy, in particular for biopsies of lesions at complex visceral sites (Criscitiello et al., 2014). Moreover, sampling tumor tissue has significant inherent limitations; tumor tissue provides a single snapshot in time and space, is subject to physical sampling bias resulting from tumor heterogeneity while the biopsy procedure itself is often costly and may be harmful. Cell-free fragments of DNA are shed into the bloodstream by cells undergoing apoptosis or necrosis, and the load of cfDNA correlates with tumor staging and prognosis (Diaz and Bardelli, 2014). cfDNA is a promising tool for accessing the tumor genome as a liquid biopsy. Whereas the prognostic impact of baseline ctDNA level might be lower than expected in patients with metastatic cancer (Madic et al., 2014), multiple blood draws over time is feasible, which would support tracking tumor dynamics in real time, as well as serving as a liquid biopsy for inaccessible metastatic tissue site. Also, although not formally demonstrated, the blood may be a reservoir for all tumor tissue in the body, thus possibly reducing sampling bias over tissue biopsies. This and other benefits to ctDNA analysis are discussed below.

Our study is among the first to report *de novo* multiplexed detection of targetable mutations in a population of metastatic cancer patients included in a prospective trial. Notably, it includes patients that (i) were not selected to have high ctDNA levels, and (ii) covered multiple tumor types. Our proof-of-concept study suggests that ctDNA analysis is a remarkable surrogate of tumor biopsy for *de novo* mutation calling, with an observed sensitivity of 97% when compared to solid tumor biopsies. Discrepancy between the two techniques was minimal: 1 mutation “missed” and 1 mutation “added” in ctDNA and, although not tested here, is likely similar to that observed between two distinct biopsies of the same tumor (Gerlinger et al., 2014). For patients with multiple targetable mutations, some relative frequencies were different between the tumor and ctDNA (e.g. in patient #5). Since our ctDNA detection technique is quantitative, these observations can be attributed to intra-tumor spatial or temporal heterogeneity. This is consistent with the hypothesis that ctDNA sampling reduces heterogeneity-related biases over single-site biopsies. In patients with detectable plasma mutations, tumor DNA represented on average 25% of the total cfDNA fraction which was comparable to published data from stage IV patients (Bettegowda et al., 2014).

Because of the diversity of tumor types and the limited size of our study, multiple tests within a single histology were rarely obtained. Our high percentage of mutation recovery from plasma DNA may have been due to our small sample size and, as a result of biological heterogeneity, may not

hold for a greater number of patient inclusions within a given tumor type. Further higher powered studies are needed to address this point.

From a clinical standpoint, the non-invasive mutational assessment through ctDNA analysis has numerous advantages over invasive biopsies with regards to patient's pain, medical costs and turnaround time. After the biopsy procedure, only three patients were discharged the same day, most patients (26/34) had to stay overnight at the hospital for post image-guided biopsy surveillance; longer stays were observed for patients who underwent surgical biopsies or as part of more extensive surgeries. In the SAFIR01 trial in metastatic breast cancer, 423 patients were included, and biopsy samples were obtained from 407 (Andre et al., 2014). Serious (grade 3 or higher) adverse events related to biopsy were reported in four (1%) of enrolled patients, including pneumothorax (grade 3, one patient), pain (grade 3, one patient), hematoma (grade 3, one patient), and hemorrhagic shock (grade 3, one patient). These complications contrast starkly to a simple and affordable blood draw.

Another interest for ctDNA highlighted here, is that in ~10–20% of patients, DNA analysis of the solid tumor is not feasible due to insufficient cellularity or failure of the biopsy procedure. In the feasibility study on the first 100 enrolled patients in the SHIVA trial, mutations, gene copy number alterations, and IHC analyses were successful in 63 (66%), 65 (68%), and 87 (92%) patients, respectively (Le Tourneau et al., 2014). At least one potentially druggable molecular abnormality was found in 38 tumors (40%). These performances are similar to those reported in the SAFIR01 study, with CGH array and Sanger sequencing feasible in 283 (67%) and 297 (70%) patients, respectively (Andre et al., 2014). A targetable genomic alteration was identified in 195 (46%) patients, most frequently in *PIK3CA* (74 [25%] of 297 identified genomic alterations). Therapy could be personalized in 55 (13%) of 423 patients. Of the 43 patients who were assessable and received targeted therapy, four (9%) had an objective response.

The NGS technique used in our study, Ion Torrent coupled to AmpliSeq chemistry, as well as other NGS platforms and sample preparation solutions are becoming increasingly standard technology in clinical laboratories worldwide. The multigene NGS test used here with broad coverage using plasma DNA provides a non-invasive option to match a variety of metastatic cancer patients to targeted therapies. As the clinical relevance of a targetable mutation is dependent on its clonal distribution, inferring somatic mutations clonal frequencies from ctDNA allelic frequencies by highly-multiplexed techniques is likely to become the next critical step in ctDNA research. Recently, multiplex PCR-based assays were applied to ctDNA to target specific coding regions in *EGFR*, *KRAS*, *BRAF*, *ERBB2* and *PI3KCA* genes in a cohort of metastatic lung cancer. Sensitivity of the test was 58% [95%CI: 43%–71%] and the estimated specificity was 87% [62%–96%] compared to tumor DNA (Couraud et al., 2014).

5. Conclusions

With techniques that are fairly standard across clinical laboratories worldwide, our results suggest that targeted sequencing of ctDNA across a panel of genes can reliably detect tumor sample point mutations *de novo* without any *a priori* information from the tumor biopsy. Fresh plasma might be proposed as an alternative tumor tissue source in molecular targeted therapy programs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2014.12.003.

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